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(54) METHODE D'OBTENTION DE CARTILAGE PAR GENIE BIOLOGIQUE

(54) METHOD FOR BIOENGINEERING CARTILAGE

(57)

A novel method for reconstructing cartilaginous tissue is provided in which isolated chondrocytes prepared into aggregates are then embedded into a hydrogel composition comprising alginate, collagen and elastin to form a chondrocyte-hydrogel construct. The construct is further cultured and prepared for transplantation in a patient to reproduce deformed or missing cartilaginous tissue which is essentially equivalent to the native tissue from which it is derived.

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(54) **METHOD FOR BIOENGINEERING CARTILAGE**

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ABSTRACT

A novel method for reconstructing cartilaginous tissue is provided in which isolated chondrocytes prepared into aggregates are then embedded into a hydrogel composition comprising alginate, collagen and elastin to form a chondrocyte-hydrogel construct. The construct is further cultured and prepared for transplantation in a patient to reproduce deformed or missing cartilaginous tissue which is essentially equivalent to the native tissue from which it is derived.

METHOD FOR BIOENGINEERING CARTILAGE

Field of the Invention

The present invention relates to bio-engineering, or reconstruction, of body tissue. In particular, the present invention relates to a novel method for reconstructing elastic cartilage that is present, for example, in auricular and epiglottal tissue, and to a novel medium for conducting such cartilage reconstruction.

Background of the Invention

Advances in the field of tissue engineering are moving realistically close to building autologous replacements for worn or damaged body parts using a patient's own cells. For cartilage replacement, one of the persistent problems remains the constraints placed on the system by a paucity of suitable starter material. Unfortunately, transplants of viable allogeneic cartilages or freshly-isolated donor chondrocytes evoke a strong immune response in the recipients that practically eliminates their use in routine plastic surgery (Moskalewski, Clin. Orthop., 272, 16-20 (1991); Hye et al., Cell Transplant., 6, 119-124 (1997); Romaniuk et al., Transpl. Immunol., 3, 251-257 (1995)). Since the factors regulating differentiation of mesenchymal precursor cells toward chondrogenesis are not well identified, differentiated autologous chondrocytes remain the only feasible starting material, despite the fact that the number of cells from biopsies of autologous elastic cartilage is usually insufficient.

The initial work of Kawiak et al. (Exp. Cell. Res., 39, 59-68 (1965)) and Moskalewski et al. (Transplantation, 3, 737-747 (1965)) followed by that of Chesterman and Smith (J. Bone Joint Surg. (Br), 50, 184-197 (1968)) revealed that chondrocytes enzymatically isolated from animal hyaline cartilages may multiply in culture and reconstruct small amounts of cartilage when transplanted intramuscularly in an autologous system. Other studies

(Wirth and Rudert, Arthroscopy, 12, 300-308 (1996); Rudert and Wirth, Orthopeade, 26, 741-747 (1997); Moskalewski (1991), *supra)*) were aimed at the establishment of a reproducible model which would allow for amplification of the original number of isolated chondrocytes in cultures to achieve a large quantity of differentiated cells capable of the efficient repair of damaged cartilages. Studies with human hyaline chondrocytes also established that the number of cells initially harvested could be greatly increased by an additional cell cultivation *in vitro* (Sittinger et al. Biomaterials, 17, 237-242 (1996); Wirth and Rudert (1996), *supra*), especially when stimulated with insulin, dexamethasone, or growth factors, such as bFGF, PDGFbb, EGF and IGF (Quatela et al., Arch. Otolaryngol Head Neck Surg., 119, 32-37 (1993); Quarto et al., Endocrinology, 138, 4966-4976 (1997)). It has also been established that facilitating cell-cell interactions in a very dense cell seeding in the initial cultures (Tacchetti et al. Exp. Cell Res., 200, 26-33 (1992); Lee et al., Dev. Dynamics, 200, 53-67 (1994)) or cell aggregation in rotating culture vessels (Duke et al., J. Cell Biochem., 51, 274-282 (1993); Kouri et al., Biocell., 20, 191-200 (1996)) may prevent chondrocyte dedifferentiation toward fibroblast-like cells in long-lasting monolayer cultures (Sittinger et al., Laryngorhinootologie, 76, 96-100 (1997); Moskalewski (1991), *supra*).

Most importantly, it has been shown that cartilages can be created in predetermined shapes and dimensions using chondrocyte transplantation on appropriate polymer templates (Kawiak et al. Exp. Cell. Res., 39, 59-68 (1965); Kim et al. Plast. Reconstr. Surg., 94, 233-237 (1994); Bujia et al., Laryngorhinootologie, 73, 577-580 (1994); Bujia et al., Laryngorhinootologie, 74, 183-187 (1995); Bujia et al., Acta Otolaryngol. (Stockh.), 115, 307-310 (1995)) Embedding of isolated hyaline chondrocytes into purified collagens (Sams et al., Osteoarthritis Cartilage, 3, 47-59 (1995); Enami et al., Cell & Tissue Culture: Laboratory Procedures, A. Doyle, J.B. Griffiths, D.G. Newell (eds.), John Wiley and Sons, TO, 1996, Module 3A:5.1-5.8; van Susante et al.,

Acta orthop. Scand., 66, 549-556 (1995)) or biodegradable gel-matrices, such as agarose (Kawiak et al. (1965), *supra*; Horwitz et al., J. Cell. Biol., 45, 434-438 (1970); Hinek et al., Acta Biol.Acad. Sci. Hung., 35, 245-258 (1984)), agarose with fleeces of bioabsorbable E 200 polymer (Rotter et al. Laryngorhinootologie, 76, 241-247 (1997) polylactic acid and polyglycolic acid (Bujia et al. (1995), *supra*) and calcium-alginate (Paige et al., Plast. Reconst. Surg., 96, 1390-1398 (1995); van Susante et al. (1995), *supra*; Guo et al., Connect. Tissue Res., 19, 277-297 (1989)) followed by their *in vivo* transplantation, has established plausible experimental models which would facilitate the reconstruction of the original cartilages for the purposes of reconstructive surgery.

Furthermore, it has been established that collagen and biodegradable gels have different advantages as carriers for hyaline chondrocyte transplantation (Enami et al. (1996), *supra*). While isolated hyaline chondrocytes embedded in collagen gels *in vitro* demonstrated a significant increase in cell numbers, they dedifferentiated into fibroblast-like cells when kept longer than 6 days in such a construct (Sittinger et al. (1997), *supra*). Chondrocytes suspended in calcium-alginate gels, on the other hand, maintained their typical chondrocyte phenotype and vigorously synthesized cartilage-specific proteoglycans, despite their slower proliferation (van Susante et al. *supra*).

In clinical situations such as anotia, microtia, and similar variants of the branchial arch syndromes, auricular elastic cartilage is both grossly deformed and in such a short supply that its remaining volume is not a sufficient source of elastic cartilage chondrocytes for reconstruction of the missing or deformed ear. Attempts to reconstruct the auricle have, therefore, been mostly based on autologous rib cartilage (Brent, Plast. Reconstruct. Surg., 90, 355-374 (1992). It has recently been shown by Cao and colleagues (Plast. Reconstr. Surg., 100, 297-302 (1997) that chondrocytes isolated from bovine hyaline cartilage, seeded onto a synthetic biodegradable polymer mesh

of polyglycolic acid/polylactic acid formed in the shape of a human auricle can reconstruct the ear-shaped cartilage when implanted into subcutaneous pockets on the dorsa of athymic mice.

Previous studies with chondrocytes isolated from animal auricular cartilages indicated that those cells may also multiply in cultures and reconstruct elastic cartilage after autologous transplantation (Hinek et al, Cell Tissue Resl, 172, 59-79 (1976); Kawiak et al., Acta Anat. (Basel), 76, 530-544 (1970). The analysis of the reconstructed elastic cartilages indicated, however, that production of the matrix macromolecules, including elastin, decline with the age of the donor, supporting the concept of "age programs" for the biosynthesis and turnover of different matrix macromolecules (Madsen et al. Dev. Biol., 96-100 (1997); Moskalewski (1991), *supra*; Moskalewski et al., Acta Anat. (Basel), 97, 231-240 (1977); Moskalewski, Connect. Tissue Res., 8, 171-174 (1981). Auricular chondrocytes isolated from human cadaver elastic cartilage have been shown to increase their *in vitro* proliferation and matrix production containing elastin when maintained in very dense cultures (Lee et al., Dev. Dynamics, 200, 53-67 (1994), or in response to basic fibroblast growth factor and transforming growth factor-beta (Quatela et al. (1993), *supra*). Elastic chondrocytes embedded in "low-melting" agarose increase their proliferative and synthetic potential in cultures and after subcutaneous transplantation. Unfortunately, agarose gels which contain the polymerized galactosugars are unable to support elastic cartilage reconstruction due to the galactosugar-dependent inhibition of the elastic fiber assembly in the newly-produced matrix (Hinek et al. (1984), *supra*).

It has become clear that, in order to conduct meaningful reconstruction of damaged cartilaginous tissues, there is a need to develop methods and biomaterials aimed at the production of autologous cartilage in amounts sufficient for reconstruction of larger cartilaginous structures.

Summary of the Invention

Accordingly, in one aspect of the present invention, there is provided a method for reconstructing cartilaginous tissue from chondroctyes, comprising the steps of:

1) preparing aggregates of said chondrocytes;
2) admixing said aggregates in a hydrogel comprising an alginate component, a collagen component and an elastin component to form a chondrocyte hydrogel construct; and
3) culturing said construct.

In another aspect, there is provided a hydrogel composition comprising an alginate component, a collagen component and an elastin component.

These and other aspects of the present invention are illustrated further in the drawings in which:

Brief Description of the Drawings

Figure 1A is a phase-contrast image (magnification 400 x) illustrating chondrocytes maintained without agitation for 72 h form a multilayer of elongated cells with fibroblast-like phenotype;

Figure 1B is a phase-contrast image, as in Fig. 1A, illustrating chondrocytes agitated for 72 h form microaggregates which maintain a round phenotype;

Figure 2A is a micrograph (magnification 100 x) of individual chondrocytes suspended in alginate gel and cultured *in vitro* for 4 weeks;

Figure 2B is a micrograph, as in Fig. 2A, of alginate-embedded aggregated cells;

Figure 2C is a micrograph, as in Fig.2A, of aggregated chondrocytes suspended in alginate gel and transplanted into the nude mice;

Figure 2D is a micrograph, as in Fig. 2A, of transplanted chondrocyte aggregates suspended in an alginate/collagen hydrogel;

Figure 2E is a micrograph, as in Fig. 2A, of aggregated porcine chondrocytes suspended in alginate/collagen/elastin hydrogel;

Figure 3 is an electron micrograph (magnification 30,000 x) of 4-week-old transplants of chondrocytes embedded in an alginate hydrogel;

Figure 4 is an electron micrograph (magnification 30,000 x) of 4-week-old transplants of aggregated chondrocytes suspended in an alginate/collagen/elastin hydrogel construct;

Figures 5A - 5D are micrographs (magnification 100 x) showing immunohistochemically-detected fibronectin (A), link protein (B), keratan sulfate proteoglycans (C), and collagen type II (D) in the extracellular matrix produced in 4 week old transplants of aggregated chondrocytes suspended in an alginate/collagen/elastin hydrogel construct;

Figure 6A is a micrograph (magnification 100 x) of the cartilaginous tissue elaborated by four-week-old transplants of aggregated human chondrocytes embedded in an alginate/collagen/elastin hydrogel construct;

Figure 6B is a micrograph, as in Fig. 6B, of neocartilage produced in twelve-week-old transplants of aggregated human chondrocytes embedded in alginate/collagen/elastin hydrogel constructs; and

Figure 7 is an electron micrograph (magnification 56,000 x) showing a fragment of fully assembled elastic capsule elaborated by transplanted aggregated human chondrocytes.

Detailed Description of the Invention

A method for bioengineering or reproducing elastic cartilage is provided in which isolated chondrocytes are formed into aggregates which, when embedded in a hydrogel, comprising alginate, collagen and elastin, can be used to reconstruct cartilaginous tissue.

Chondrocytes, enzymatically isolated from an autologous cartilaginous source in a manner well-known to those of skill in the art, are prepared into chondrocyte aggregates by constant agitation in a suitable growth medium

under appropriate growth conditions. An example of a suitable growth medium is HEPES-buffered Ham's F12 medium containing 10% fetal calf serum. The cells are subjected to such agitation for a period of time to allow growth and, thus, aggregation to occur, for example, a period of time of about 48-72 hours. This method of preparing chondrocyte aggregates utilizes the biological phenomenon of homoaggregation, allowing for selection against contaminating fibroblasts which attach to plastic, promoting selection of the most potent chondrocytes and preventing chondrocyte dedifferentiation.

Once chondrocyte aggregates have been formed and collected, they are resuspended in growth medium and admixed with a hydrogel composition comprising alginate, collagen and elastin components.

The alginate component of the hydrogel comprises alginate dissolved in water or other appropriate buffer. Alginate itself is a biopolymer comprising the monomers, mannuronate and guluronate linked by 1->4 glycosidic linkages. Different alginate gels contain different proportions of the mannuronate and guluronate monomers, depending on the physical and chemical properties of the desired alginate gel. When dissolved in water or other buffer, the alginate is formed into a gel by addition of di-valent cations such as sodium, calcium or copper. Accordingly, the alginate gel used to prepare the hydrogel of the present invention may vary with respect to the proportion of its monomer components. However, in order to be effective in the present hydrogel to catalyze reconstruction of elastic cartilage, the composition of the alginate used must be such that it forms a biodegradable gel to allow growth of cartilaginous tissue from chondrocyte aggregates. Generally, the amount of alginate required to form a suitable gelatinous product is in the range of about 1-4% alginate when combined with water or buffer.

The collagen component appropriate for inclusion in the present hydrogel composition includes autologous or allo human collagen, as well as highly purified zeno collagen isolated from non-human mammalian sources,

for example, bovine, porcine or equine sources. Although all types of collagen are suitable for use in the present hydrogel, collagen type I and collagen type II are the most preferred types.

To prepare the hydrogel, the alginate component is admixed with the collagen component to form a biodegradable gel that functions to stimulate chondrocyte cells to grow and reproduce. In order to attain efficient chondrocyte growth, the alginate and collagen components are preferably combined in a ratio ranging from 2:1 alginate/collagen to 1:2 alginate/collagen. In this regard, it is important to avoid a hydrogel comprising excess alginate, resulting in a hydrogel which is too gelatinous to biodegrade adequately. It is also important to avoid a hydrogel comprising excess collagen so as to minimize the risk of adverse immunologic response in circumstances of transplant. The most preferred ratio of alginate to collagen in the present hydrogel is a 1:1 alginate/collagen admixture.

The elastin component of the hydrogel comprises a heterogeneous mixture of soluble elastin-derived peptides which range in molecular weight from 5-70 Kd. This elastin component is prepared from insoluble elastin polymer by any one of a number of methods. For example, soluble elastin-derived peptides may be prepared from elastin by alkaline hydrolysis in ethanol as described in detail in Kornefeld-Dourllain and Robert (Bull. Soc. Chim., 50:579-771 (1968)), by hydrolysis in concentrated formic acid, or by enzymatic digestion with pancreatic elastase. The elastin component of the hydrogel may be derived from either human or non-human mammalian sources, the most practical of which is the latter. The elastin component is significant to stimulate chondrocyte reproduction, to preserve chondrogenic phenotype and, thus, prevent dedifferentiation. In order to provide an effective hydrogel, the elastin component is preferably present in the hydrogel in an amount ranging from 0.1-2 mg/ml of the hydrogel.

The hydrogel composition, comprising the alginate, collagen and elastin components as set out above, is combined with chondrocyte aggregates

to form a chondrocyte hydrogel construct, the cellular density of which is about 5-10% of the total hydrogel volume, i.e. a cellular density of approximately 1-2 x 10^6 cells per milliliter of hydrogel.

The construct is then cultured further to allow the cells the opportunity to adapt to the hydrogel and to encourage vigorous cellular multiplication. The hydrogel construct may be formed into beads, in a manner well-known to those of skill in the art and described in more detail in the examples herein, for further culturing. Once chondrocyte growth is established in the construct, the construct may be prepared for surgical implantation into a patient to reproduce elastic cartilage that is essentially equivalent to the native cartilage from which it is derived. In this regard, the construct can be molded into a preferred shape for transplantation, for example, into the shape of an ear or part thereof to reconstruct missing or deformed auricular cartilage. It can also be used in the reconstruction of facial defects, molded appropriately for inplantation or injected directly into the tissue.

Embodiments of the present invention are exemplified by the following specific examples which are not to be construed as limiting.

Example 1 - Preparation of Chondrocytes

With parental consent and Institutional Ethics Committee approval, small fragments of auricular cartilage were harvested from 3 children (<1 year of age) at the time of surgery for extirpation of pre-auricular tags. The resected tags contained small quantities of elastic cartilage. Similarly, auricular cartilage samples were obtained from 3 older children (4-7 years) undergoing staged ear reconstruction for microtia. Elastic cartilage samples were also harvested under appropriate anesthesia from the ears of 6 juvenile female Yorkshire White pigs. These specimens, stripped of their perichondrium, were stored in phosphate-buffered saline (PBS) containing (200 U/ml of penicillin and streptomycin and 200 μg/ml of amphotericin B

(Antibiotic-antimycotic, Gibco-BRL®) and then used for cell isolation within 2 h of harvest.

Chondrocyte isolation was achieved by digestion of diced cartilage with bacterial Collagenase type 1 (Life Technologies, Gibco-BRL®) dissolved in PBS (3 mg/ml w/v). Total digestion of the initial cartilages (5-10 g diced to 2-3 mm pieces suspended in 10 ml buffer) at 37°C in a rotary agitator was achieved in 16-18 h. Isolated cells were counted on a haemocytometer and concomitantly checked for viability by trypan blue exclusion.

Isolated chondrocytes were resuspended in HEPES-buffered Ham's F12 medium, containing 10% fetal calf serum and antibiotics/antimycotics, and then seeded onto 100 mm tissue culture dishes at an initial density of 1 x 10^6 cells/dish. The cells were maintained at 37°C in a humid, 5% CO_2 atmosphere for 10 days. Parallel, similarly-plated cells were placed on a horizontal shaker and constantly agitated for 48-72 h. While non-agitated cells formed a monolayer culture, the agitated cells were prevented from settling and attaching to the plastic, forming aggregates of undamaged floating chondrocytes. Chondrocytes growing in monolayer cultures for 10 days were harvested by trypsinization (15 min in 0.1% Trypsin-EDTA solution obtained from Gibco-BRL), while the 48- or 72-hour-old aggregates were collected by slow centrifugation.

Comparison between isolated elastic chondrocytes kept in a monolayer culture and aggregated chondrocytes confirmed that cell shape plays an important role in expression of chondrogenic phenotype. Just 4-8 hours of agitation led to formation of small chondron-like aggregates containing 4-10 chondrocytes, which grew to larger clusters (20-70 cells) in 72-h cultures (Fig.1B). The aggregated chondrocytes maintained their round shape and, after 72 h in culture, were surrounded by a visible extracellular matrix. Isolated elastic chondrocytes maintained without agitation quickly attached to the plastic dishes, multiplied, and formed the multilayer culture of elongated and fibroblast-like cells (Fig. 1A) which produced only little extracellular matrix.

Example 2 - Preparation of Hydrogel Constructs

The composite hydrogel constructs were made using a 50/50 mixture of sodium alginate (UP MVG, Pronova Biopolymer®) and bovine Type 1 collagen gel (Vitrogen 100® Collagen Corporation, Palo Alto, Ca.), with or without an additional supplement of a heterogeneous mixture of soluble elastin-derived peptides, k-elastin (Elastin Products, Owensville. MO). To make the composite hydrogels, a 3% solution (W/V) of low viscosity sodium alginate was prepared and sterilized by autoclaving at 120°C for 30 minutes. Once cool, the alginate was mixed 1:1 with a sterile solution of purified, pepsin-solubilized bovine, dermal collagen dissolved in 0.012 N HCl which was then neutralized and kept on ice. Kappa-elastin was dissolved in sterile distillate water and added to the alginate-collagen hydrogel mixture prior to gelation at a final concentration of 2 mg/ml of gel.

Example 3 - Immurement of Chondrocytes in Hydrogel Constructs

Suspensions of the single chondrocytes harvested from the primary 10-day-old monolayer cultures or pre-cultured microaggregates were resuspended in Ham's F-12 medium and then readily entrapped in alginate hydrogel or in composite constructs. This was done by mixing the hydrogel with suspensions of free chondrocytes or aggregates to a concentration of about 5-10% of the total volume of the hydrogel, gently vortexing to ensure homogeneity and then forming hydrogel/cell mixture into beads by expelling droplets from a syringe (fitted with a 27 gauge needle) into 102 mM calcium chloride solution. The calcium chloride solution can vary in concentration from 20 - 120 mM. After 10 minutes, cured beads of 100 to 500 ml volume were then suspended in F-12 medium and incubated at 37°C.

Quadruplicates of each hydrogel construct, containing either human or porcine, free or aggregated elastic chondrocytes, were then further cultured for a period of four weeks or surgically implanted beneath the dorsal skin of

twelve juvenile, female athymic (NMRI nu/nu) nude mice for a period of four or twelve weeks. The immunohistochemical detection of cartilage specific matrix components in 4 week old cultures and in 4 week old transplants of aggregated porcine chondrocytes embedded in alginate (A), alginate/collagen (B), alginate/collagen/k-elastin (C) constructs was as set out in Table 1 below.

Table 1

	Cultures			Transplants		
	A	B	C	A	B	C
Link Protein	++	++	+++	+++	++++	++++
Keratan Sulfate PG	++	++	+++	++	++++	++++
Fibronectin	++++	++	++	++	++	++
Type II Collagen	++	++++	++++	++	++++	++++
Elastin	+	++	+++	++	++	++++

Non-aggregated chondrocytes maintained their secretory phenotype when suspended in alginate gel and cultured for 4 weeks. The extracellular material produced by those non-aggregated cells was similar to matrix of hyaline cartilage and did not contain elastin detectable by the Movat's stain (Fig. 2A) nor immunohistochemistry (data not shown). In contrast, the alginate-embedded aggregated chondrocytes produced matrix which contained elastin, the hallmark of the original elastic cartilage (Fig 2B).

Both non-aggregated and aggregated porcine chondrocytes suspended in alginate gel further amplified their chondrogenic properties after transplantation to the nude mice and produced greater quantities of the elastin-containing extracellular matrix than they did in culture. However, extracellular matrix produced in four week-old transplants of the alginate embedded chondrocytes contained irregularly scattered elastin clusters (Fig.2C and Fig.3). This contrasted with transplanted chondrocyte aggregates suspended in the mixture of alginate and collagen type I that produced islets of elastic cartilage, which contained elastin deposited in the form of long fibers (Fig. 2D). The total amount of the reconstructed elastic cartilage containing immuno-detectable fibronectin, link protein, keratan sulfate proteoglycans, and

collagen type II (Fig 5A-D), and particularly the amount of properly assembled elastic fibers and capsules produced by transplanted aggregated chondrocytes, was most impressive when the alginate/collagen-containing hydrogel was further enriched with k-elastin (Table I, Fig. 2E and Fig. 4). Morphometric analysis also shows that the areas occupied by dark brown structures detected by Movat's stain perfectly overlapped with the elastin detected by immunostaining performed on the adjacent serial sections.

The immunohistochemical detection of cartilage specific matrix components in 4 and 12 week old transplants of aggregated human chondrocytes embedded in alginate/collagen/k-elastin constructs was as set out in Table 2 below.

Table 2

	4 weeks	12 weeks
Link Protein	++++	++++
Keratan Sulfate PG	++++	++++
Fibronectin	++	+
Type II Collagen	+++	++++
Elastin	++++	++++

Histological analysis of transplants of the aggregated human chondrocytes embedded in hydrogel containing alginate, collagen, and k-elastin also show that the tissue elaborated in four-week-old transplants closely resembles the original elastic cartilage. It was surrounded by the perichondrium-like structure and contained only small islets of the original hydrogel indicating that this biodegradable supporting material had been replaced by the newly-produced extracellular matrix (Fig 6A). The neocartilage was even larger in volume in the twelve-week-old transplants and did not show signs of dedifferentiation or degeneration (Fig. 6B). In fact, the overall organization of this cartilage, immunohistochemistry with a panel of antibodies to the major cartilage matrix components (Table 2), and especially the presence of fully-assembled elastic fibers (Fig. 7) further confirms that cartilage reconstructed from the human aggregated chondrocytes embedded in

a construct containing alginate, collagen and k-elastin resembled the native auricular cartilage even more than that of porcine origin as described above.

Description of the Procedures used to analyze cultures and transplants

Histochemistry: All specimens scheduled for light microscope evaluation were fixed in 10% buffered formalin and routinely prepared for paraffin sections. The fast polychromic staining was carried out using modified Movat's method (Musto, J. Histotechnology, 9, 173-174 (1986)) which yields collagen stained yellow, proteoglycans green, and elastin dark brown. The dark brown structures detected by this fast Movat's stain always overlapped with elastin detected by the immunostaining with an anti-elastin antibody (Elastin Products, Owensville. MO) performed on the adjacent serial sections.

Immunohistochemistry: Samples of cultures and transplants were fixed in 4% paraformaldehyde. The cryostate sections were then subjected to immunostaining. The following monospecific and affinity purified antibodies were used to detect cartilage matrix components: polyclonal antibody to tropoelastin (Elastin Products, Owensville. MO), an MB 140 monoclonal antibody to cell-associated fibronectin (Chemicon, Tamecula, CA), monoclonal antibody to collagen Type II, monoclonal antibody to keratan sulfate, and polyclonal antibody to cartilage proteoglycan link protein (the latter three antibodies were a generous gift from Dr. Robin Poole of the Joint Diseases Laboratory at the Shriner's Hospital for Crippled Children, Montreal, PQ). The immunoreactions were visualized with either goat anti-mouse (GAM) or goat anti-rabbit (GAR) fluorescein-conjugated secondary antibodies (ZYMED, San Francisco, CA). The nuclei were counterstained with red propidium iodide (Sigma, St. Louis, MO).

Quantitative assessment of extra cellular matrix: Morphometric analysis of sections immunostained with antibodies recognizing matrix components, as well as those stained with the Movat's method was performed using an Olympus AH-3 microscope attached to a CCD camera (Optronix) and a computer-generated video analysis system (Image-Pro Plus software, Media

Cybernetics, Silver Spring, MD. USA). In each experimental setting fifty low power fields (40 x) of 10 sections from three separate cultures or transplants were analyzed and the area occupied by the particular immunodetectable component quantified. The abundance of each immunodetectable component was then expressed as a percentage of the entire analyzed field and graded as: 5% (+), 5-10 (++), 10-20% (+++), more than 20% (++++) (see Tables 1 and 2).

Electron Microscopy: 12-week-old explants containing porcine and human chondrocytes were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer containing 0.2% tannic acid, postfixed with 1% osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in Epon, a technique which assures high contrast of partially polymerized elastin (black) and fully polymerized elastin (gray), when thin sections are stained with uranyl acetate and lead citrate (Hinek et al., Cell Tissue Res., 172, 59-79 (1976); Thyberg and Hinek, Cell Tissue Res., 180, 341-356 (1977)). It has previously been documented that a highly contrasted amorphous material detected by this method can be selectively immuno-stained with an anti-elastin antibody detected with the immuno-gold (Hinek and Rabinovitch, J. Cell Biol, 126, 563-574 (1994)).

We claim:

1. A method for reconstructing cartilaginous tissue from chondroctyes, comprising the steps of:

1) preparing aggregates of said chondrocytes;

2) admixing said aggregates in a hydrogel comprising an alginate component, a collagen component and an elastin component to form a chondrocyte hydrogel construct; and

3) culturing said construct.

2. A method as defined in claim 1, wherein said alginate component comprises alginate dissolved in water and admixed with divalent cations.

3. A method as defined in claim 2, wherein the alginate component comprises about 1-4% alginate.

4. A method as defined in claim 1, wherein said collagen component is selected from the group consisting of collagen type I and collagen type II.

5. A method as defined in claim 1, wherein the alginate and collagen components of the composition are combined in an amount ranging from a ratio of about 1:2 alginate component to collagen component to a ratio of about 2:1 alginate component to collagen component.

6. A method as defined in claim 1, wherein said elastin component comprises soluble peptides ranging in molecular weight from 5-70 kilodaltons.

7. A method as defined in claim 1, comprising about 0.1-2 mg/ml of the elastin component.

8. A method as defined in claim 1, wherein said chondrocyte aggregates are admixed with said hydrogel at a concentration of about 5-10% of the total volume of said hydrogel.

9. A method as defined in claim 1, further comprising the step of transplanting the cultured construct into a patient at a site requiring reconstruction of tissue.

10. A hydrogel composition comprising an alginate component, a collagen component and an elastin component

11. A hydrogel composition as defined in claim 10, wherein said alginate component comprises alginate dissolved in a water and admixed with divalent cations.

12. A hydrogel composition as defined in claim 11, wherein the alginate component comprises about 1-4% alginate.

13. A hydrogel composition as defined in claim 10, wherein said collagen component is selected from the group consisting of collagen type I and collagen type II.

14. A hydrogel composition as defined in claim 10, wherein the alginate and collagen components of the composition are combined in an amount ratio ranging from a ratio of about 1:2 alginate component to collagen component to a ratio of about 2:1 alginate component to collagen component.

15. A hydrogel composition as defined in claim 10, wherein said elastin component comprises soluble peptides ranging in molecular weight from 5-70 kilodaltons.

16. A hydrogel composition as defined in claim 10, comprising about 0.1-2 mg/ml of the elastin component.

Fig. 1

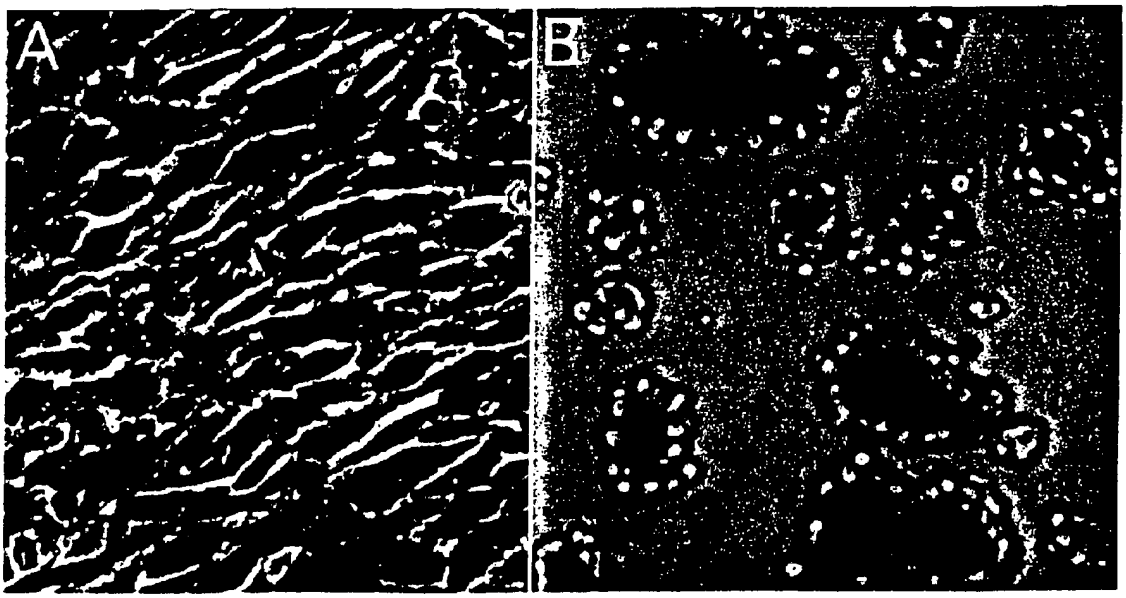
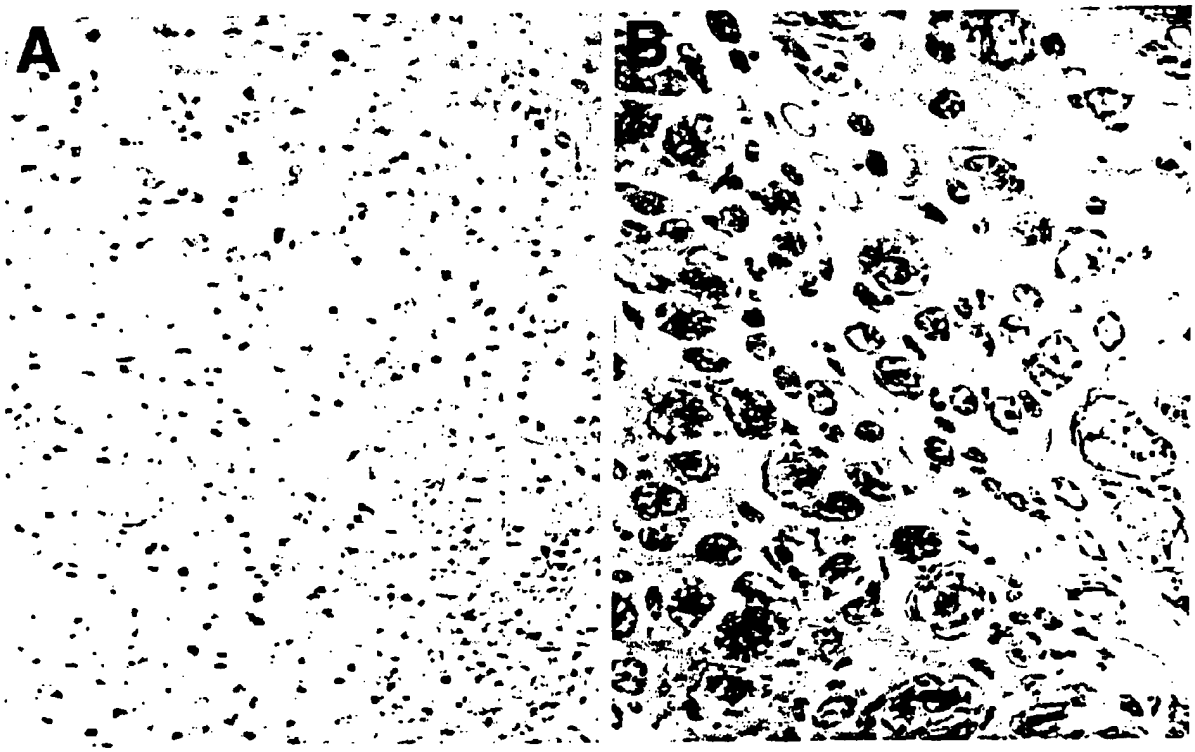


Fig. 2



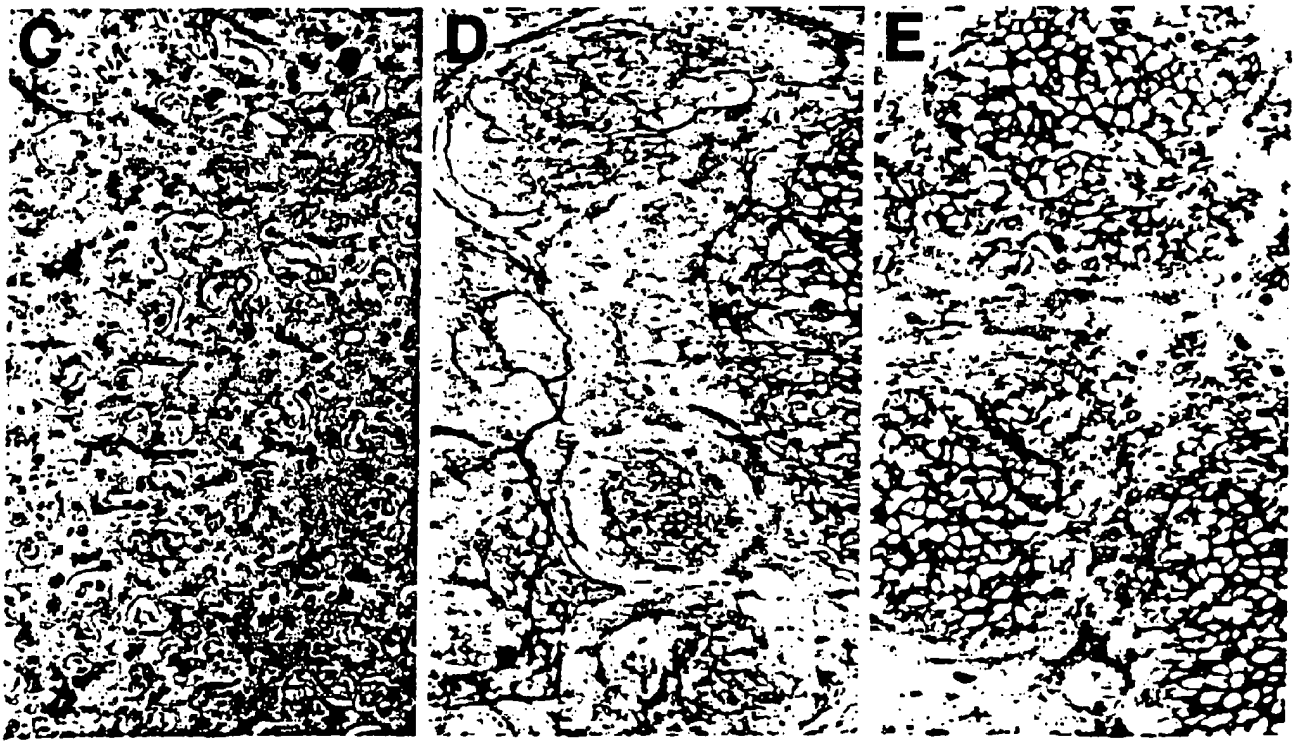


Fig. 3

Fig. 4

Fig. 5

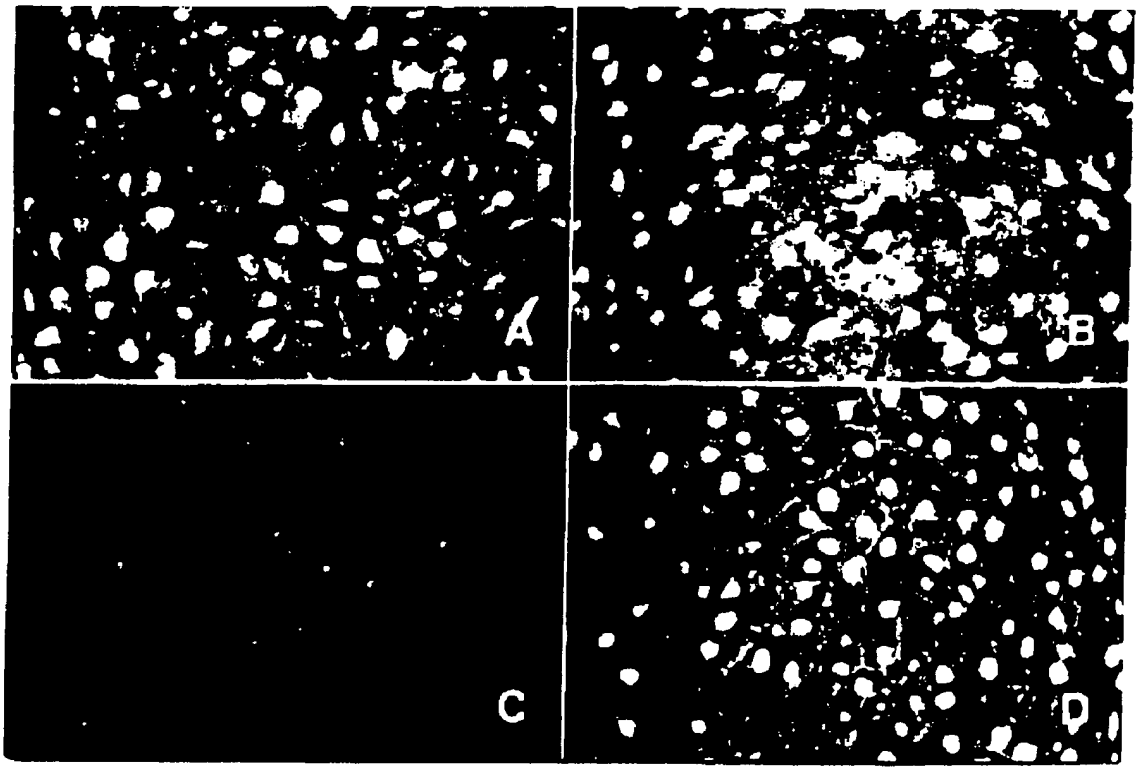


Fig. 6

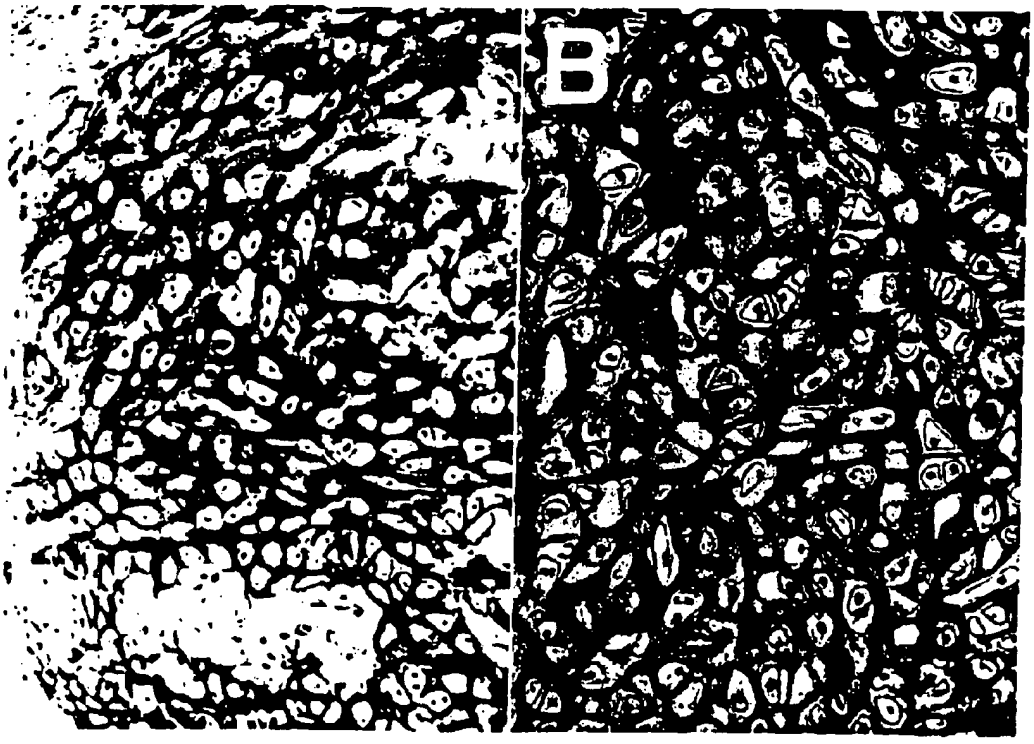


Fig. 7